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Who Is a Carrier? Detection of Unsuspected Mutations in 21-Hydroxylase Deficiency

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Congenital adrenal hyperplasia due to 21-hydroxylase deficiency is a common autosomal-recessive disorder. During our routine genotyping of affected individuals and their relatives using allele-specific oligonucleotide hybridization and single-strand conformational polymorphism analysis, we identified two families each segregating three mutations. In both families, a mutation known to be associated with 21-hydroxylase deficiency was identified in healthy individuals but was not detected in the propositus. The propositus in family 1 was shown to be a homozygous carrier for G at nucleotide 655, which alters the splice acceptor site at exon 3. The propositus in family 2 carried the same splicing mutation on the maternal allele and a gene deletion/conversion on the paternal allele. In both families, other clinically unaffected relatives carried the Q318X mutation in exon 8. If molecular diagnostic studies had been limited to the mutation carried by the propositi, relatives would have been misinformed regarding their status as carriers or mildly affected individuals. The findings in these two families emphasize the high frequency of alleles causing 21-hydroxylase deficiency in the population.
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KEY WORDS: **congenital adrenal hyperplasia, 21-hydroxylase deficiency, adrenal steroidogenesis, adrenal gland**

INTRODUCTION

Congenital adrenal hyperplasia is a common autosomal-recessive inborn error of steroidogenesis. Impaired biosynthesis of cortisol within the adrenal gland leads to increased adrenocorticotropin (ACTH) and adrenal androgen secretion. Decreased 21-hydroxylase activity accounts for approximately 90% of the cases of congenital adrenal hyperplasia. The frequency of mild 21-hydroxylase deficiency exceeds that for cystic fibrosis, neurofibromatosis 1, Duchenne muscular dystrophy, and sickle-cell anemia [Speiser et al., 1985]. The incidence of classical 21-hydroxylase deficiency is very high in the Yupik Eskimos, while the incidence of nonclassical 21-hydroxylase deficiency is high in the Ashkenazi Jewish population.

The range of clinical manifestations extends from glucocorticoid and mineralocorticoid deficiencies with prenatal virilization of female fetuses to postpubertal virilization. Traditionally, patients are categorized into one of three major classifications. Salt-losing 21-hydroxylase deficiency is characterized by glucocorticoid and mineralocorticoid deficiencies. Affected females present at birth with ambiguous external genitalia, while affected males present within the first 3 weeks of life in adrenal crisis. In simple virilizing 21-hydroxylase deficiency, premature pubarche occurs before age 5 years. Premature pubarche manifests as premature occurrence of sexual hair, acne, adult-type body odor, accelerated growth velocity, and advanced skeletal maturation; affected males may have phallic enlargement with prepubertal testicular volume. The symptoms of nonclassical or mild 21-hydroxylase deficiency, i.e., hirsutism, irregular menses, and infertility in postpubertal women, are secondary to excessive adrenal androgen secretion [Kohn et al., 1982]. This broad phenotypic spectrum presumably reflects the level of 21-hydroxylase activity [Morel and Miller, 1991].

Through linkage studies, the gene encoding 21-hydroxylase activity, CYP21, was mapped to the class III HLA region on the short arm of chromosome 6 [Dupont et al., 1977; White et al., 1986]. This gene lies in duplication with a highly homologous nonfunctional pseudogene, CYP21P [Higashi et al., 1986]. The structure of both genes is comparable: each is approximately 3.4 kb in length and contains 10 exons. The pseudogene seems to function as a reservoir for deleterious mutations [Wedell and Luthman, 1993].

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The molecular basis of 21-hydroxylase deficiency has been well characterized [Morel et al., 1989; White and New, 1992; Helmberg, 1993]. Many of the previously identified deleterious mutations in the functional gene are attributed to gene conversion events with the pseudogene. One commonly detected mutation is a gene conversion event at nucleotide 655 in the second intron, in which a normally polymorphic nucleotide (A or C) is converted to a G which affects the splicing of this intron. In two in vitro studies, the predominant effect of this "splicing mutation" was activation of a cryptic splice acceptor site which altered the reading frame, leading to a premature stop codon in the third exon [Higashi et al., 1988; Owerbach et al., 1992]. Another previously recognized deleterious mutation is a C to T transversion at nucleotide 1994, leading to conversion of codon 318 from glutamine to a stop codon [Globerman et al., 1988; Urabe et al., 1990]. In transient transfection studies using COS cells, 21-hydroxylase activity was determined to be 10% with the splicing mutation, and 0% with Q318X relative to a normal functional gene [Higashi et al., 1991].

As part of our investigation of phenotype/genotype relationships in affected individuals, we offer DNA analysis for the 21-hydroxylase gene to extended relatives [Siegel et al., 1995]. Members of two families were found to carry an additional mutation associated with 21-hydroxylase deficiency (Q318X) which was not carried by the propositi. These findings illustrate the complexities involved in the genetic analysis of 21-hydroxylase deficiency, and underscore the high frequency of mutant CYP21 alleles in the general population.

MATERIALS AND METHODS

Patients

The patients have been followed by the Endocrine Division of the Children's Hospital of Pittsburgh. One patient presented neonatally for evaluation of ambiguous genitalia. The second patient presented at age 8 years with premature pubarche. Following informed parental consent and patient assent, blood samples were obtained from the propositus and as many relatives as feasible for human leukocyte antigen (HLA) haplotype and molecular genetic analysis. This protocol is approved by the Human Rights Committee of the Children's Hospital of Pittsburgh.

ACTH Stimulation Tests

Blood samples were obtained prior to and 30 min following an intravenous bolus of Cortrosyn, 0.25 mg, over a 1-min period. Cortrosyn stimulation tests were performed in as many relatives as available. Progesterone, 17-hydroxyprogesterone, and androstenedione were determined using double antibody radioimmunoassay (RIA) kits with ^{125}I-labeled steroids (ICN Biomedicals Inc., Carson, CA). Respective interassay coefficients of variation (n = 20) were 12.6%, 11.0%, and 9.2%. Cortisol was assayed using a solid-phase ^{125}I RIA (Diagnostic Products Corporation, Los Angeles, CA), with interassay coefficient of variation (n = 20) of 6.1%.

Allele-Specific Oligonucleotide Hybridization (ASOH)

To amplify exons 1–8 of CYP21, three different polymerase chain reaction (PCR) amplication reactions, containing 1 μg genomic DNA, 20 pmol of each primer, 2.0 mM $MgCl_2$, 200 μM dNTP, 10 μl 10 × PCR buffer (Perkin Elmer, Foster City, CA), and 1.9 units *Taq* polymerase (Perkin Elmer Foster City, CA) in a total volume of 100 μl, were utilized (Fig. 1). Primers 2F (5′-TCG GTG GGA GGG TAC CTG AA-3′) and 2R (5′-CCA GAG CAG GGA GTA GTC TC-3′) amplified exons 1–3 of CYP21. Primers 3F (5′-ACC TGT CCT TGG GAG ACT AC-3′) and 3R (5′-CGG TAG CAT CAC TGG CTG TG-3′) amplified exons 3–6 of CYP21. Primers 4F (5′-GAG GGA TCA CAT CGT GGA GAT-3′) and 4R (5′-GCT TTC CTC ACT CAT CCC CAA-3′) amplified exons 7 and 8 of CYP21. PCR conditions were 94°C for 1 min and 64°C for 4 min for 30 cycles. With these primers, only CYP21 was amplified.

Following phenol-chloroform extraction, 2 μl of PCR product were spotted onto Hybond-N+ membranes

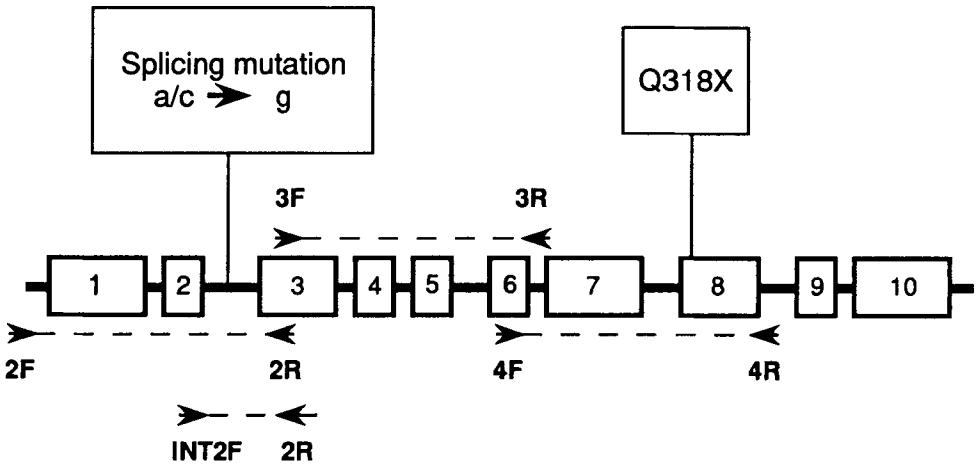

Fig. 1. Diagram of CYP21. Locations of the splicing mutation at nucleotide 655 and C→T at nucleotide 1994 are indicated. Primers used for PCR amplification for ASOH and SSCP analyses are indicated. Primers 2R, 3F, and 4F, specifically, amplify only CYP21.

(Amersham, Arlington Heights, IL). Membranes were soaked in 0.4 N NaOH for 20 min followed by a brief wash in 2 × SSC. Membranes were prehybridized for at least 2 hr at 42°C in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, and herring sperm DNA (10 μg/ml). Overnight hybridization at 42°C with ^{32}P end-labelled oligonucleotide probes for both the normal and mutant sequences (1×10^6 dpm/ml) was performed. Membranes were initially washed in 6 × SSC, 0.5% SDS at 42°C for 40 min, followed by a stringent wash.

Utilizing the PCR product amplified by primers 2F and 2R, ASOH to detect the splicing mutation at nucleotide 655 in the second intron was performed with differential hybridization probes: 665A (5′-AGCCCCCAACTCCTCCTG-3′; stringent wash temperature, 60°C); 655 C (5′-AGCCCCCACCTCCTCCTG-3′; 60°C); and 655G (5′-AGCCCCCAGCTCCTCCTG-3′; 62°C). The PCR product generated with primers 3F and 3R was probed to detect I172N with the probes: Ile^{172} (5′-AGGTAACAGATGATGCTG-3′; 52°C) and Asn^{172} (5′-AGGTAACAGTTGATGCTG-3′; 52°C). The differential hybridization probes for the PCR product generated by primers 4F and 4R were: Val^{281} (5′-GCCATGTGCACGTGCCCTTC-3′; 70°C); Leu^{281} (5′-GCCATGTGCAAGTGCCCTTC-3′; 68°C); 1761 (5′-CGTGAAGCAAAAAAACCACGGCC-3′; 70°C); i1761T (5′-CGTGAAGCAAAAAAAACCACGGCC-3′; 72°C); Glu^{318} (5′-TAGCTCCTCCTGCAGTCGCTG-3′; 68°C); $STOP^{318}$ (5′-TAGCTCCTCCTACAGTCGCTG-3′; 66°C); Arg^{356} (5′-AACGGGCCGCAGGCGCA-3′; 60°C); and Trp^{356} (5′-AACGGGCCACAGGCGCA-3′; 58°C).

When a patient appeared to be a homozygous carrier for a specific mutation, but only one parent was a heterozygous carrier for the mutation, the patient was considered a heterozygous carrier of a gene conversion event [Speiser et al., 1992a].

Single-Strand Conformational Polymorphism Analysis (SSCP)

For the four recognized mutations in exons 7 and 8, SSCP and heteroduplex analyses were performed as previously described [Siegel et al., 1994]. Primers used to amplify intron 2 were Int2F (5′-TGGGCAGACTTTGCTGGCAGAC-3′) and 2R. Gel composition and electrophoresis conditions were identical to those previously described [Siegel et al., 1994]. Conditions for SSCP analysis using primers 3F and 3R were identical to those previously described, including restriction enzyme digestion of the PCR product with *BglI* [Siegel et al., 1994].

RESULTS

Family 1

The proposita was referred for the evaluation of genital ambiguity and an elevated 17-hydroxyprogesterone level on newborn screening. On ASOH, her DNA sample hybridized only to G655 and not to either of the probes for the normal alleles C655 and A655, indicating likely homozygosity for this splicing mutation in intron 2 (Fig. 2). Samples from her mother, father, and maternal grandfather hybridized to the oligonucleotide probes for G655 and A655 (Fig. 2). Thus, patient 1 is a homozygous carrier for the splicing mutation, while her parents and maternal grandfather are heterozygous carriers for this mutation. DNA samples from her paternal grandmother, maternal grandmother, paternal uncle, and paternal first cousins hybridized only to the probe for A655 and not for C655 or G655 (Fig. 2). These individuals, i.e., her paternal grandmother, maternal grandmother, paternal uncle, and paternal cousins, carry the normal sequence and would appear not to carry the mutant alleles present in the propositus. Hence, these individuals a priori would be considered noncarriers of 21-hydroxylase deficiency. The DNA sample from the proposita's paternal grandfather hybridized only to G655. Based on the alleles inherited by his sons, the paternal grandfather appears to carry the splicing mutation on one allele and a gene deletion/conversion on his other allele (Fig. 2).

Allele-specific oligonucleotide hybridization and SSCP analyses were performed on the DNA samples obtained from the patient and her relatives to assess for I172N, V281L, i1761T, Q318X, and R356W mutations. Allele-specific oligonucleotide hybridization showed that the paternal grandmother, paternal uncle, and paternal cousins are heterozygous carriers for the Q318X mutation (Fig. 2). For these individuals, results of SSCP analysis were concordant with the results obtained by ASOH. By ASOH and SSCP analyses, the patient and her parents do not carry the Q318X mutation. HLA haplotype and SSCP analyses confirm that the father and paternal uncle inherited different CYP21 alleles from their mother (Fig. 2).

The 17-hydroxyprogesterone (17-OHP) incremental elevations on the ACTH stimulation tests (Table II) were consistent with carrier status for the father, paternal uncle, and maternal grandfather. For the paternal grandmother, the 17-hydroxyprogesterone incremental rise was not elevated. The 17-hydroxyprogesterone incremental response of the paternal grandfather, 1,322 ng/dl, was consistent with heterozygote or mildly affected status.

Family 2

The proposita in family 2 was referred for evaluation of premature pubarche at age 8 years. Her basal 17-hydroxyprogesterone level was elevated at 1,665 ng/dl. Sixty min following ACTH infusion, her 17-hydroxyprogesterone level was 3,621 ng/dl. Her 60-min incremental elevation (1,956 ng/dl) was elevated. By ASOH, her DNA sample hybridized only with the probe for G655 (Fig. 3). Samples from her mother, brother, and maternal grandmother hybridized to the oligonucleotide probes for both A655 and G655 (Fig. 3). DNA samples obtained from the proposita's father and maternal grandfather hybridized only to the oligonucleotide probe for A655 (Fig. 3). Her mother, brother, and maternal grandmother appear to be heterozygous carriers of the splicing mutation, while her father appears to be a heterozygous carrier of a gene conversion/deletion. The proposita carries the splicing mutation on the maternal allele and a gene conversion/deletion on the paternal allele.

On ASOH and SSCP analyses, her father and brother were found to carry the Q318X mutation (Fig. 3). By

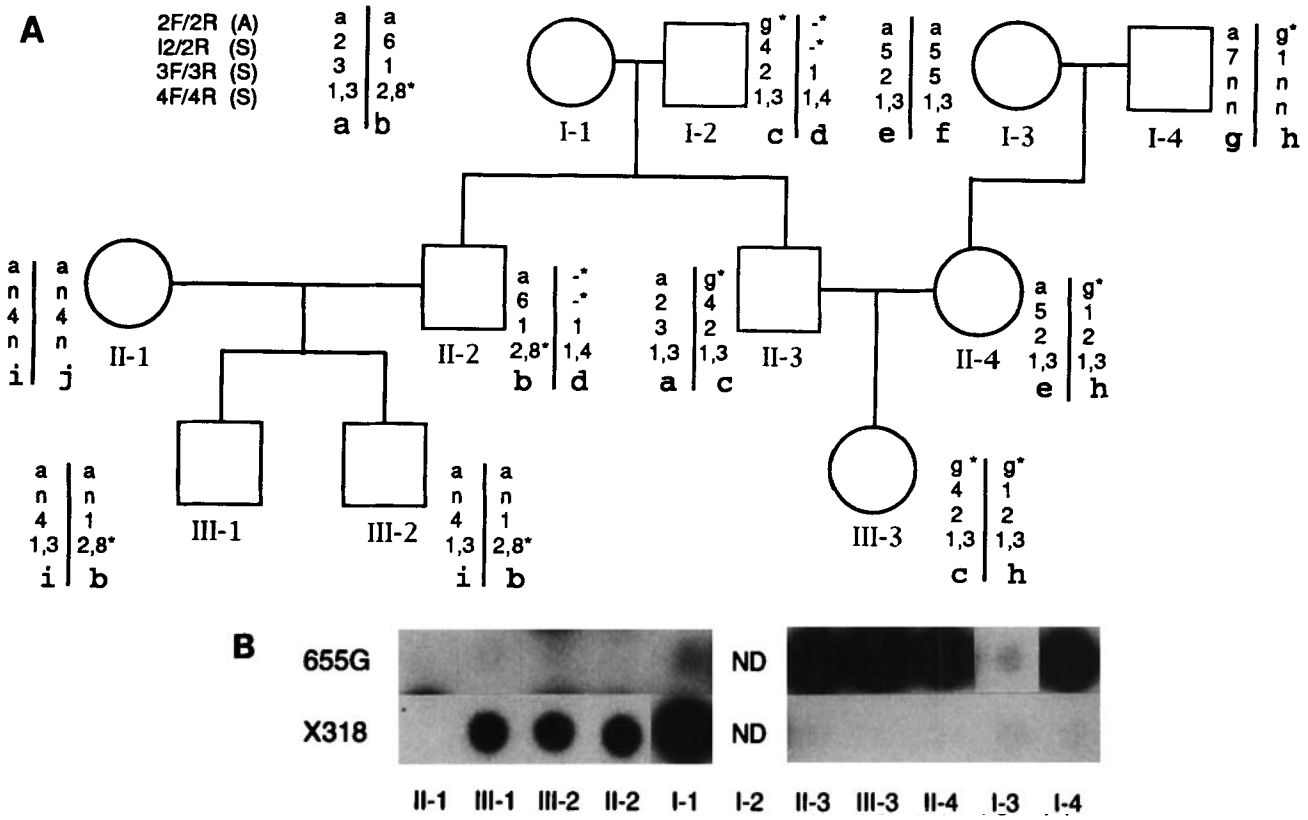

Fig. 2. Pedigree and allele-specific oligonucleotide results from family 1. **A:** Diagram representing family 1 is indicated. Below each marker, there is an identification number for each individual. Results from ASOH and SSCP analyses (in phase) are indicated by each marker in the pedigree. Each unique SSCP conformer was labelled by number, as indicated; n, not done. Letter beneath SSCP results defines the extended HLA haplotype (Table I). For individuals I-1 and I-2, extended haplotypes for HLA loci are presumed based on the haplotypes of other family members. **B:** Allele-specific oligonucleotide hybridization results. Results of ASOH with probes hybridizing to sequences with splicing mutation or X318 are shown. Results of ASOH with probes hybridizing to wild-type sequences are described in the Results section.

ASOH and SSCP analyses, the propositus does not carry the Q318X mutation (Fig. 3). The HLA haplotypes and SSCP analysis indicate that the brother inherited the same maternal CYP21 allele as did the proposita (Table I). However, the sibs inherited different paternal CYP21 alleles.

On the SSCP analysis of exons 7 and 8 of CYP21 in the midportion of the gel, the proposita possessed a unique conformer which she could only have inherited from her father, because her mother does not have the same conformer (Fig. 3). This conformer was not inherited by her brother; he inherited two separate conformers consistent with inheritance of a different paternal allele. These results showing noninheritance of the paternal allele for intron 2 and inheritance of the paternal allele for the downstream exons 7 and 8 provide strong evidence for a gene conversion affecting the 5′ portion of CYP21 on the paternal allele inherited by the proposita.

The 17-hydroxyprogesterone incremental elevations in the father, mother, and brother are consistent with decreased 21-hydroxylase activity (Table II). Assessment of the brother's skeletal maturation showed that his bone age was consistent with his chronologic age.

DISCUSSION

Both families requested genetic counseling. If molecular diagnostic studies had been limited to the splicing mutation identified in the propositae, the Q318X mutation in both families would have escaped detection. The Q318X mutation causes classical salt-losing 21-hydroxylase deficiency with 0% enzyme activity in transient transfection expression studies [Higashi et al., 1991; Speiser et al., 1992a]. Furthermore, both families would have received inaccurate information regarding their risks for progeny with 21-hydroxylase deficiency. In addition, while HLA haplotypes confirmed parental status, these families did not carry the specific haplotypes with known genetic linkage disequilibrium for congenital adrenal hyperplasia, i.e., HLA-B47 and salt-losing 21-hydroxylase deficiency [Dupont et al., 1981].

For family 1, the paternal grandmother, paternal uncle, and paternal cousins would have been informed that they were not carriers of a CYP21 gene associated with congenital adrenal hyperplasia. However, molecular analysis showed that these individuals carry the Q318X mutation on one CYP21 allele, which differs from the CYP21 allele with the splicing mutation in-

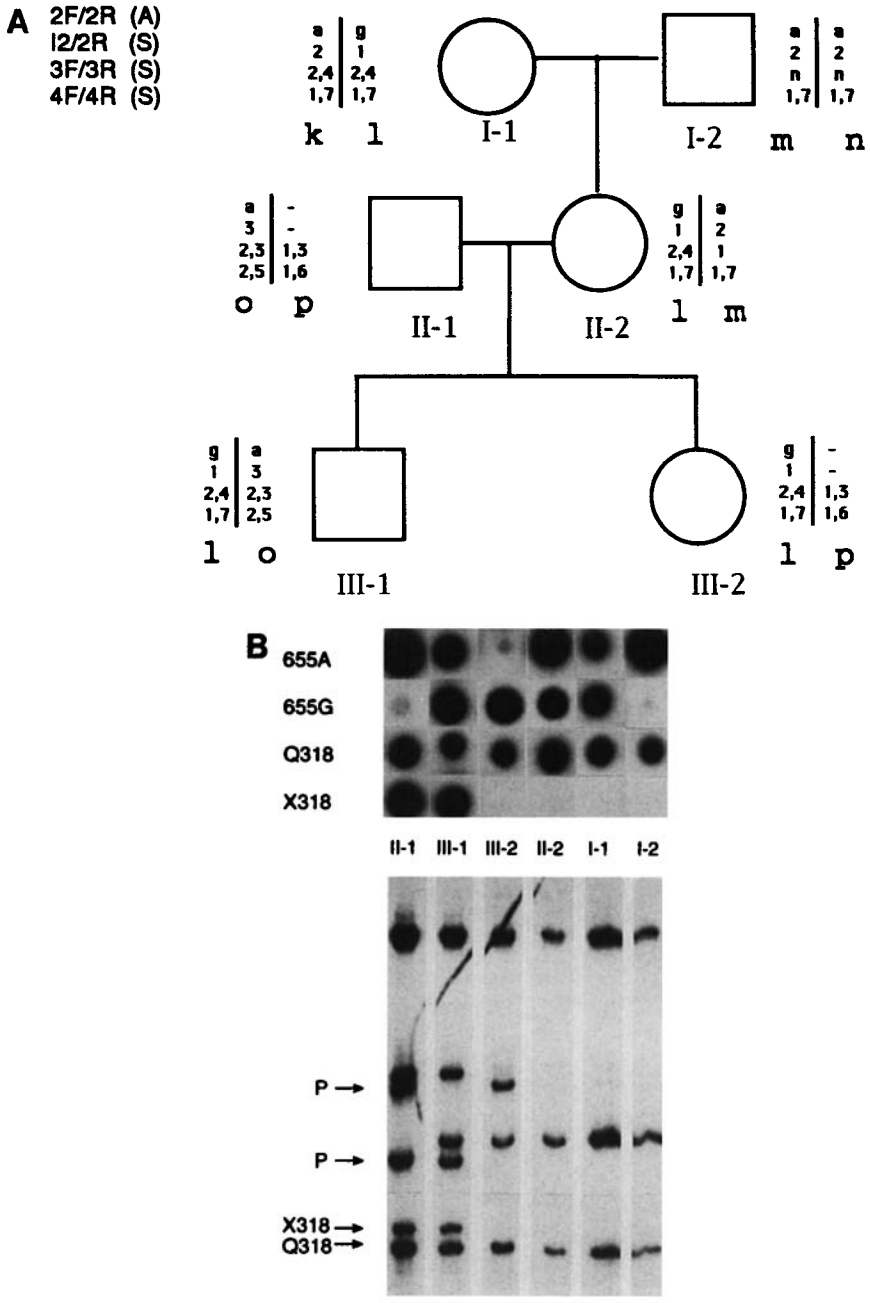

Fig. 3. Pedigree, allele-specific oligonucleotide hybridization, and single-strand conformational polymorphism (SSCP) analysis from family 2. **A:** Pedigree, with identification numbers and extended haplotypes, is shown at top. Individual results from ASOH and SSCP analyses are indicated by allele. **B:** Allele-specific oligonucleotide hybridization and SSCP results. ASOH results for four probes are shown above the SSCP gel. 665A and Q318 indicate probes for normal CYP21 sequences, while 655G and X318 indicate mutant sequences. Individuals III-2 and II-2 have a conformer in the lower portion of the gel representing Q318. Individuals II-1 and III-1 have two conformers representing Q318 and X318. Individuals II-1, III-1, and III-2 have additional bands in the midportion of the gel representing polymorphisms. Individual III-2 has a conformer which she could only have inherited from her father (II-1), because her mother does not have that particular conformer. Based on these additional conformers, two different paternal alleles can be distinguished. Upper portion of the gel showing V281 conformers, and the middle of the gel, which does not contain any bands, are not shown.

herited by the propositus. For the paternal uncle, the 17-hydroxyprogesterone incremental response provides biochemical corroboration of his carrier status [Lee and Gareis, 1975]. The maternal grandmother had a normal 17-hydroxyprogesterone incremental elevation, which can be seen in some heterozygotic carriers [New et al., 1983]. Based on his genotype, the paternal grandfather should have had 21-hydroxylase deficiency. His

TABLE I. Key to Extended Haplotypes Used in Figures*

Extended haplotype	HLA-A	HLA-B	Nucleotide 655	Codon 318	HLA-DR
Family 1					
a	A24	B7	A	Q318	DR1
b	A2	B50	A	X318	DR7
c	A25	B18	G	Q318	DR15
d	A1	B62	Del	Q318	DR12
e	A3	B7	A	Q318	DR15
f	A2	B40(60)	A	Q318	DR8
g	A1	B57	A	Q318	DR15
h	A32	B40(60)	G	Q318	DR11
i	A2	B39	A	Q318	DR15
j	A26	B13	A	Q318	DR11
Family 2					
k	A24	B39	A	Q318	DR8
l	A2	B51	G	Q318	DR8
m	A31	B60	A	Q318	DR4
n	?	?	A	Q318	?
o	A2	B50	A	X318	DR7
p	A2	B62	Del	Q318	DR13

* Column at left represents extended haplotype in the figure. Each column shows individual haplotype with HLA-A, HLA-B, CYP21 (nucleotide 655 and codon 318), and HLA-DR as indicated. For nucleotide 655, A represents normal sequence and G indicates splicing mutation. For codon 318, Q318 represents normal sequence and X318 indicates C to T transversion at nucleotide 1994, changing codon 318 from Gln to stop.

hormonal profile and asymptomatic phenotype are consistent with late-onset 21-hydroxylase deficiency in a male.

The parents in family 2 would have been counseled that their son is a heterozygotic carrier for the splicing mutation. Detection of the Q318X mutation altered his clinical classification from carrier to mildly affected. The 17-hydroxyprogesterone incremental responses of the parents and brother indicate mildly decreased 21-hydroxylase activity. Such responses are consistent with both nonclassical 21-hydroxylase deficiency and heterozygotic carrier status [New et al., 1983]. The normal-statured father denies premature puberty. In this instance, the molecular genetic information showed that the father and brother are mildly affected individuals rather than heterozygous carriers. The molecular genotype would predict more severe phenotypes than observed for the father and brother.

TABLE II. Hormone Levels*

Individual	Basal 17-OHP	Progesterone	17-OHP
Family 1			
I-1	30	21	71
I-2	110	84	1,322[c]
I-3	50	25	127
I-4	165	70	297[c]
II-1[a]	96	1	70
II-2	147	3	221[c]
II-3	129	75	560[c]
II-4[b]	36	14	154
Family 2			
II-1	139	201[c]	567[c]
II-2[a]	261	40	360[d]
III-1	95	84	477[c]
Healthy men			
Mean	138	37.1	79.8
SD	35	33.3	45.8
Healthy women			
Mean	45	32.2	93.0
SD	32	18.8	45.8

* Basal 17-hydroxyprogesterone levels are listed in first column. Progesterone and 17-hydroxyprogesterone incremental responses 30 min following ACTH stimulation are listed in second and third columns. Units for hormone determinations are ng/dl. Values for healthy adults are indicated at bottom of table body.
[a]Luteal phase of menstrual cycle.
[b]One month postpartum.
[c]>2.57 SD above mean for healthy adult men.
[d]>2.57 SD above mean for healthy adult follicular-phase women.

Both families are of German and Irish ethnic background. Family 2 also has maternal American Indian ancestry. In the German population, classical salt-losing 21-hydroxylase deficiency has been associated with the extended haplotype A3, B47, DR7, while nonclassical disease has been linked to A33, B14, DR1 [Knorr et al., 1985]. In the Irish population, the extended haplotype A3, B40(60), DR1 accounts for 26% of affected alleles [Sinnott et al., 1991]. These extended haplotypes were not associated with mutant alleles in our families.

Both the intron 2 splicing mutation and the Q318X mutation are associated with moderate-to-severe impairment of 21-hydroxylase activity [Higashi et al., 1991]. For example, the splicing mutation was determined to be the molecular basis of classical salt-losing 21-hydroxylase deficiency among the Yupik Eskimos [Speiser et al., 1992b]. Thus, based on the molecular genotypes of the propositi, i.e., the paternal grandfather in family 1 and the brother in family 2, we would anticipate that these 4 individuals would have salt-losing or simple virilizing 21-hydroxylase deficiency. Yet, they differ markedly in the age of clinical presentation, degree of virilization, and magnitude of salt loss. The clinical variability illustrated by these individuals emphasizes the enigma of phenotype/genotype interpretations in the splicing mutation associated with 21-hydroxylase deficiency. Preliminary sequence analysis of intron 2 has demonstrated additional sequence alterations. Such sequence alterations could influence splicing efficiency by activating alternative splice acceptor sites, or by enhancing splicing at the usual site [Ohshima and Gotoh, 1987]. Further investigation is necessary to clarify the molecular basis of this phenotypic heterogeneity.

Disease frequency for classical 21-hydroxylase deficiency is 1/8,000. Recent results from newborn screening for congenital adrenal hyperplasia in Switzerland are similar (1/8,045) [Scherz et al., 1993; Pang and Clark, 1993; Torresani et al., 1994]. When all ethnic groups are combined, the reported disease frequency for mild 21-hydroxylase deficiency is 1/111. The findings in these two families emphasize how frequent the alleles associated with 21-hydroxylase deficiency are in the population. The report of a single CYP21 allele simultaneously carrying four deleterious mutations stresses the importance of segregating alleles [Wedell et al., 1994]. Reliable genetic counseling necessitates thorough molecular genetic analysis.

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